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Olfactory Neuronal Cell Lines Generated by Retroviral Insertion of the *n-myc* Oncogene Display Different Developmental Phenotypes

K.P.A. MacDonald, A. Mackay-Sim, G.R. Bushell, and P.F. Bartlett
Faculty of Science and Technology, Griffith University, Nathan, Queensland, Australia (K.P.A.M., A.M.-S., G.R.B.); Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia (P.F.B.)

Being genetically homogeneous, clonal cell lines are potentially important for investigating many aspects of cellular differentiation. We describe here the creation of clonal cell lines by immortalization of neuronal precursor cells from the adult mouse olfactory epithelium. Unlike neurons elsewhere in the vertebrate nervous system, the olfactory sensory neuron can be replaced throughout the lifespan of the animal. However, little is known about the molecular aspects of olfactory neurogenesis. Continuous cell lines were generated by retroviral transduction of the *n-myc* proto-oncogene into the mitotically active basal cells of the olfactory epithelium which give rise to the sensory neuron. Twenty-one clonal cell lines were produced which could be divided into three distinct morphological classes: one with flat, epithelial-like cells only; another with round, flat, and bipolar cells; and a third with large flat and large bipolar cells. These morphological classes had different patterns of intermediate filament expression, as shown by immunocytochemistry and immunoblot analysis. All cells in all cell lines expressed the intermediate filament protein vimentin. Most bipolar cells, but not other cell types, expressed neurofilament protein and in one morphological class the bipolar cells co-expressed neurofilament and glial fibrillary acidic protein. Several cell lines expressed mRNA for OMP, a marker of mature olfactory sensory neurons, and G_{OLF}, a guanine nucleotide binding protein involved in olfactory sensory transduction. It is concluded that these cell lines were immortalized from sensory neuron precursors late in the lineage pathway. Other cell lines appear to have been immortalized at earlier stages in the lineage pathway. These cell lines therefore provide useful tools for the investigation of neuronal differentiation and sensory transduction in the olfactory epithelium.

Key words: immortalized cell lines, olfactory neuron, G_{OLF}, OMP, neurofilament, GFAP

INTRODUCTION

Clonal cell lines are genetically homogeneous populations of cells which are powerful tools in the analysis of cell lineage and cellular differentiation (Lendahl and McKay, 1990; Ryder et al., 1990). Immortalization of neurons and the production of neuronal cell lines can be achieved with the introduction of oncogenes via retroviral insertion during cell division of neuronal precursors (Cepko, 1988). This technique has been used successfully to immortalize neuronal precursors from embryonic mouse neuroepithelium (Bartlett et al., 1988) and neural crest (Murphy et al., 1991), embryonic rat cerebellum (Frederiksen et al., 1988), embryonic mouse brain (Trotter et al., 1989), and postnatal mouse olfactory bulb and cerebellum (Ryder et al., 1990). This report describes the immortalization of neuronal precursors from the adult mouse olfactory epithelium.

In adult mammals neurogenesis continues in the olfactory epithelium where the sensory neurons are continually produced from a pool of mitotically active stem cells (Moulton et al., 1970; Graziadei and Monti Graziadei, 1979). The retention of this "embryonic" capability in the adult may be essential to the maintenance of the sense of smell, since neuronal damage in the olfactory epithelium is probably inevitable due to the direct exposure of the sensory neuron dendrite and cilia to the external environment. Neurogenesis therefore supplies a pool of immature neurons which are available to replace any sensory neurons which are damaged by chemical, physical or infectious agents (Breipohl et al., 1986; Hinds et al., 1984; Mackay-Sim and Kittel, 1991; Carr and Farbman, 1992).

An exact description of the cell lineage of neurogenesis and differentiation of olfactory sensory neurons is

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Address reprint requests to Dr. Alan Mackay-Sim, Faculty of Science and Technology, Griffith University, QLD 4111, Australia.

not yet determined. It begins with an asymmetrical division of a stem cell followed by one or more symmetrical divisions within a precursor population (Mackay-Sim and Kittel, 1991) whose terminal division produces the immature sensory neuron (Calof and Chikaraishi, 1989). The identities of the stem and precursor cells are not known but they are contained within the basal cells of the olfactory epithelium whose population is composed of "dark" or "horizontal" cells and "light" or "globose" cells (Andres, 1966). The globose basal cells express neural cell adhesion molecule (NCAM; Calof and Chikaraishi, 1989; Miragall et al., 1988) and can give rise to sensory neurons (Graziadei and Monti Graziadei, 1979). The horizontal basal cells express keratin (Calof and Chikaraishi, 1989; Suzuki and Takeda, 1991) and can give rise to NCAM-positive cells in vitro (Satoh and Takeuchi, 1995) although this was not observed in vivo (Caggiano et al., 1994). The basal cells thus provide at least two immunologically distinct populations of mitotically active cells which could be subject to retroviral immortalization.

The proto-oncogene *n-myc* is useful for immortalizing neuronal precursors (Bartlett et al., 1988; Murphy et al., 1991). The *myc* family of proto-oncogenes contains three well defined members, *c-myc, n-myc,* and *l-myc.* Although the normal functions of these proto-oncogenes remain unclear, their expression during development appears to be tissue- and stage-specific (Semsei et al., 1989). Expression of *n-myc* in vivo is associated with neuronal development and differentiation (Zimmerman et al., 1986), and there is evidence that the activated *n-myc* is associated with neuroblastoma formation (Alt et al., 1986). Although *n-myc* cannot transform cells on its own, it can render cells immortalized if it is constitutively expressed. This can be facilitated by linking the gene to a strong transcriptional promoter, the technique used in this study to immortalize neuronal precursor cells in the olfactory epithelium of the adult mouse.

MATERIALS AND METHODS

Preparation of Olfactory Epithelial Cells

Experiments were carried out on 3 month old mice (Quackenbush, Central Animal Breeding House, Herston, Queensland; and C57/Bl, Walter and Eliza Hall Institute for Medical Research Animal House, Victoria, Australia). Four days prior to epithelial culture, the olfactory bulbs were removed bilaterally. Under Nembutal anaesthesia (pentobarbitone sodium, Boehringer Ingelheim, Ingelheim, Germany, 0.2 g/Kg b.w.), a dorsal craniostomy was performed above both olfactory bulbs using a dental burr, and the bulbs were removed by suction using a pulled and fire-polished pasteur pipette. Four days later, the animals were sacrificed by cervical dislocation and the olfactory mucosa was removed from the nasal septum and the turbinates. Pieces of olfactory epithelium were collected in Dulbecco's modified Eagles medium (DMEM, Gibco-BRL, Gaithersburg, MD) containing 1% foetal calf serum (FCS; CSL, Melbourne, Australia). The epithelium was then teased away from the underlying stroma using 26 gauge needles, with the aid of a dissection microscope. The resulting fragments of olfactory epithelium were washed twice in DMEM containing 1% FCS and dissociated by trituration through a siliconized pasteur pipette. The resulting cell suspension, which contained mainly single cells and some small clumps of cells, was diluted to 1×10^4 cells/ml.

n-myc Infection of Olfactory Epithelial Cells

The pMPZenSVNeo(*n-myc*) vector used for the immortalization of the olfactory cell lines was constructed by insertion of the 4160 bp *Hin*dII/*Sac*II fragment of the murine *n-myc* gene (DePinho et al., 1986) into the *Xho*I site of the pMPZenSV*Neo* vector DNA (Hariharan et al., 1988). This fragment contains exons 2 and 3, 820 bp of IVS-1 and 10 bp of the 3′ untranslated end. Virus stocks were produced by infection of Ψ-2 cells with the shuttle vector pZen carrying the N-myc retrovirus and *neo R* gene (Bartlett et al., 1988; Bernard et al., 1989). Ψ-2 cells producing pMPZenSVNeo(*n-myc*) virus were plated in 24 well Linbro plates (ICN, Costa Mesa, CA), permitted to grow to confluency and irradiated with 2800 Rad. The medium was removed from the irradiated monolayers, and replaced with 500 μl of the olfactory epithelial cell suspension. After 3 days in culture, the medium in each well was replaced with fresh DMEM containing 1 g/L glucose, 3.7 g/L sodium bicarbonate, 10% FCS and 400 μg/ml Geneticin (G418, Gibco-BRL). The medium was replaced every 3–4 days until the cultures became confluent with the infected olfactory epithelium cells. By this time, the cultures had been depleted of the irradiated Ψ-2 virus producing cells which had died during the culture period. When the cultures were confluent, the medium was aspirated, and the cells were incubated in 100 ml of trypsin-versene solution (CSL). When the cells began to lift from the substratum, 1 ml of DMEM containing 10% FCS was added to each well, and the cells were recovered by centrifugation at 1,500*g*. The cells were resuspended in DMEM containing 10% FCS, and cloning was performed by single cell micromanipulation. Single cells were drawn into a capillary tube, and individual cells were transferred to single wells in Terasaki plates (Nunc, Roskilde, Denmark). The cultures were grown to confluency, trypsinized and transferred to 96 well plates (Linbro, ICN). The cultures were again grown to confluency, trypsinized and transferred to 24 well plates. Once in 24 well plates, cultures

were initially split 1:3 until the lines were robust enough to recover from further dilution, after which time they were transferred to 25 cm^2 flasks (Nunclon, Nunc).

Nissl Staining

The cells were fixed in ice cold methanol for 15 min, rinsed in distilled water and covered with 0.1% cresyl violet in water. The cells were stained for 15 min, rinsed in distilled water and allowed to air dry.

Immunocytochemistry

Cells were seeded onto glass coverslips at a density of 1×10^4 cells/coverslip, and maintained in DMEM containing 10% FCS. After 48 hr incubation the coverslips were washed and fixed with ice-cold methanol at −20°C for 20 min. After fixation, the coverslips were washed and non-specific binding sites were blocked by incubating cells in 1% FCS for 60 min at room temperature. The coverslips were then incubated with primary antibody (diluted in PBS containing 1% FCS) for 60 min with at room temperature. The antibodies used were: anti-neurofilament (rabbit polyclonal antibody to 150 kD subunit: Chemicon, Temecula, CA; 1:400), anti-vimentin (mouse monoclonal antibody; Boehringer-Mannheim, Mannheim, Germany; 10 mg antibody/ml) and anti-GFAP (rabbit polyclonal antibody; Dakopatts, Carpinteria, CA; 1:100). For controls, primary antibodies were omitted and the cells were incubated in normal serum derived from the same species as the primary antibody (1:400). The coverslips were washed and the cells were incubated for 60 min at room temperature with a flouresceine-conjugated (Sileaus Laboratories, Sussex, UK) secondary antibody diluted 1:50 in PBS containing 1% FCS. The choice of secondary antibody was appropriate to the species in which the primary antibody was raised. The coverslips were washed, mounted in PBS: glycerol (1:9 v/v), containing 2.6% 1,4-diaszobicyclo-(2,2,2)-octane (DABCO; Merck, Darmstadt, Germany) and the secondary antibody was visualized using fluorescence microscopy.

SDS Gels and Immunoblotting

For each cell line, cells from one confluent 80 cm^2 flask were harvested by scraping, washed once in cold PBS, and resuspended in 300 ml of buffer containing 30 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA and 1 mM phenylmethylsulphonyl flouride (PMSF). The cells were homogenized and the nuclei removed by centrifugation at 2,000*g* for 10 min. Supernatants were collected and protein concentration estimated using the colorimetric Coomassie Brilliant Blue G-250 binding assay (Bradford, 1976). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using a BioRad Protean II minigel apparatus (BioRad, Hercules, CA, USA). Proteins (20 mg) were first run through a 3% acrylamide stacking gel, then separated in 7.5% (for neurofilament and GFAP) or 15% (for OMP) acrylamide separating gel. Transfer of proteins from SDS-polyacrylamide gels onto nitrocellulose membranes (BioRad) by horizontal electrophoresis was performed using a semi-dry transfer apparatus (Multiphor II; Pharmacia, Milwaukee, WI). The nitrocellulose filters (blots) were transferred to blocking solution (5% low fat skim milk powder in PBS). The blots were blocked overnight at 4°C, followed by a 1 hr incubation in the primary antibody (diluted in blocking solution) at room temperature. The antibodies were: neurofilament (rabbit polyclonal, Chemicon, 1:400), GFAP (rabbit polyclonal, Dakopatt, 1:100) and OMP (goat polyclonal, gift of Dr Frank Margolis, 1:500). The blots were washed once for 5 min and twice for 15 min each wash in blocking solution and then incubated for 1 hr at room temperature in secondary antibody conjugated with horseradish peroxidase (HRP, BioRad). The secondary antibodies were anti-rabbit (for neurofilament and GFAP staining) or anti-goat (for OMP staining) IgG, diluted 1:500 in blocking solution. After removal from the secondary antibody solution, the blots were washed once for 5 min and twice for 15 min each wash in Tris-buffered saline (20 mM Trizma base, 137 mM NaCl, 3.8 mM HCl; pH 7.6) prior to performing colorometric detection of HRP. HRP-conjugated secondary antibody was visualized using 4-chloro-1-naphthal as a substrate.

RNA Isolation

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was isolated from cells and tissue using the method described previously (Gonda et al., 1982).

Northern Blot Analysis

Electrophoresis of RNA through formaldehyde containing gels was performed as described previously (Fourney et al., 1988). Briefly, 25 μl of RNA sample buffer was added to 5 μl of RNA (20 μg total RNA or 5 μg mRNA), and the samples were incubated at 65°C for 15 min. Ethidium bromide (EtBr, 1 μl of a 1.0 mg/ml solution, Boehringer Mannheim) was introduced into each sample, the samples were loaded onto a gel made up of 1% agarose (Sigma, St. Louis, MO), 0.66 M formaldehyde, and 1 × MNE (20 mM 3-(N-morpholino) propanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate) buffer. The RNA was electrophoresed submerged in 1 × MNE buffer at 80 V at room temperature. RNA was transferred to nylon membranes (Hybond-N; Amersham International, Amersham, UK) and hybridized to ^{32}P-

Fig. 1. Scanning electron micrograph of olfactory mucosa after removal of the olfactory epithelium. The basement membrane remains essentially undisturbed (lower left) following removal of the olfactory epithelium, some of which is shown here still attached (upper right). Scale bar = 100 μm.

labelled random hexamer-primed probes. Following 18 hr hybridization, filters were washed under stringent conditions and exposed to x-ray film (Fuji Photofilm Co., Tokyo, Japan) with an intensifying screen (Dupont, Sydney, Australia) at −70°C.

RESULTS

Efficacy of Dissection and Preparation of Olfactory Epithelial Cells

Following the removal of olfactory epithelial cells, the remaining olfactory mucosa was examined using scanning electron microscopy. The micrographs clearly show that although a large proportion of the olfactory epithelial cells was removed during scraping, the submucosa remained essentially undisturbed (Fig. 1).

Morphology of Olfactory Cell Lines

After the dissociated olfactory epithelium was cultured on the irradiated virus-producing Ψ-2 cell lines, small epithelial-like colonies were evident within the monolayer within 3 days. At this time, G418 was added to the culture medium to select cells infected with the retrovirus. After G418 treatment for 7 days many of the epithelial-like colonies remained viable. By 10–14 days of G418 treatment none of the irradiated Ψ-2 cells remained, leaving only the G418-resistant cells which continued to proliferate. Distinct morphological differences between the colonies were apparent within 14 days of addition of G418.

After cloning by single cell manipulation 21 clonal cell lines of divers morphology were generated. These immortalized cell lines were grouped into three broad morphological classes. Class I cell lines contained rapidly dividing, flat, adherent, epithelial-like cells with a cell body diameter of approximately 40–50 μm (Fig. 2A,B). Class II cell lines were also rapidly dividing cell lines, comprised of cells with three distinct morphologies: flat, adherent epithelial-like cells similar to those seen in Class I; bipolar cells with short processes which extended up to 50 μm; and round refractile cells (Fig. 2C,D). Class III cell lines contained two morphological cell types: large, flat epithelial-like cells with a cell body diameter of approximately 150–200 μm and bipolar cells with processes which extended up to 200 μm (Fig. 2E,F).

Intermediate Filament Expression in the Olfactory Cell Lines

Each morphological class of cell lines showed a different pattern of expression of the three intermediate filament proteins, neurofilament, glial fibrillary acidic protein (GFAP) and vimentin. This expression was examined with immunocytochemistry and immunoblot analysis. Examples of the immunocytochemistry are presented in Figure 3. Examples of the immunoblot analysis are presented in Figure 4.

Immunocytochemistry of Class I cell lines either showed no neurofilament expression or a few immunoreactive cells. Neurofilament expression in Class I cells lines could not be confirmed by immunoblot analyses (Fig. 4A). The expected size of the neurofilament protein identified with this antibody is 150 kD (Shaw, 1991). In contrast, all cell lines within Classes II and III contained some cells which were immunoreactive with the neurofilament antibody (Fig. 3A–F). This immunoreactivity was confirmed in the immunoblots of all protein preparations derived from the Classes II and III (Fig. 4A).

No Class I cell lines were GFAP-positive. The expected size of the GFAP protein identified with this antibody is 50 kD (DeArmond et al., 1983). Cells within Class II cell lines contained some cells which were immunoreactive with the GFAP antibody (Fig. 3G–J) but this was not confirmed by immunoblot analysis. Cells in Class III cell lines were immunoreactive with the GFAP antibody (Fig. 3H,K) a result confirmed by immunoblot analysis (Fig. 4H,K). All cell lines from all morphological classes were strongly vimentin-positive and all cells within each cell line stained positively (Fig. 3K,L).

Olfactory Marker Protein and G_{OLF} Expression

In the immunoblot analysis of protein extracts, none of the cell lines tested contained any proteins which exhibited OMP-like immunoreactive protein (Fig. 5) whereas the olfactory epithelium showed a strong signal corresponding to the correct molecular weight for OMP (19 kD), as expected (Fig. 5). In contrast, a Northern analysis detected OMP mRNA in four cell lines of Class II and in the olfactory epithelium and bulb (Fig. 6A). In

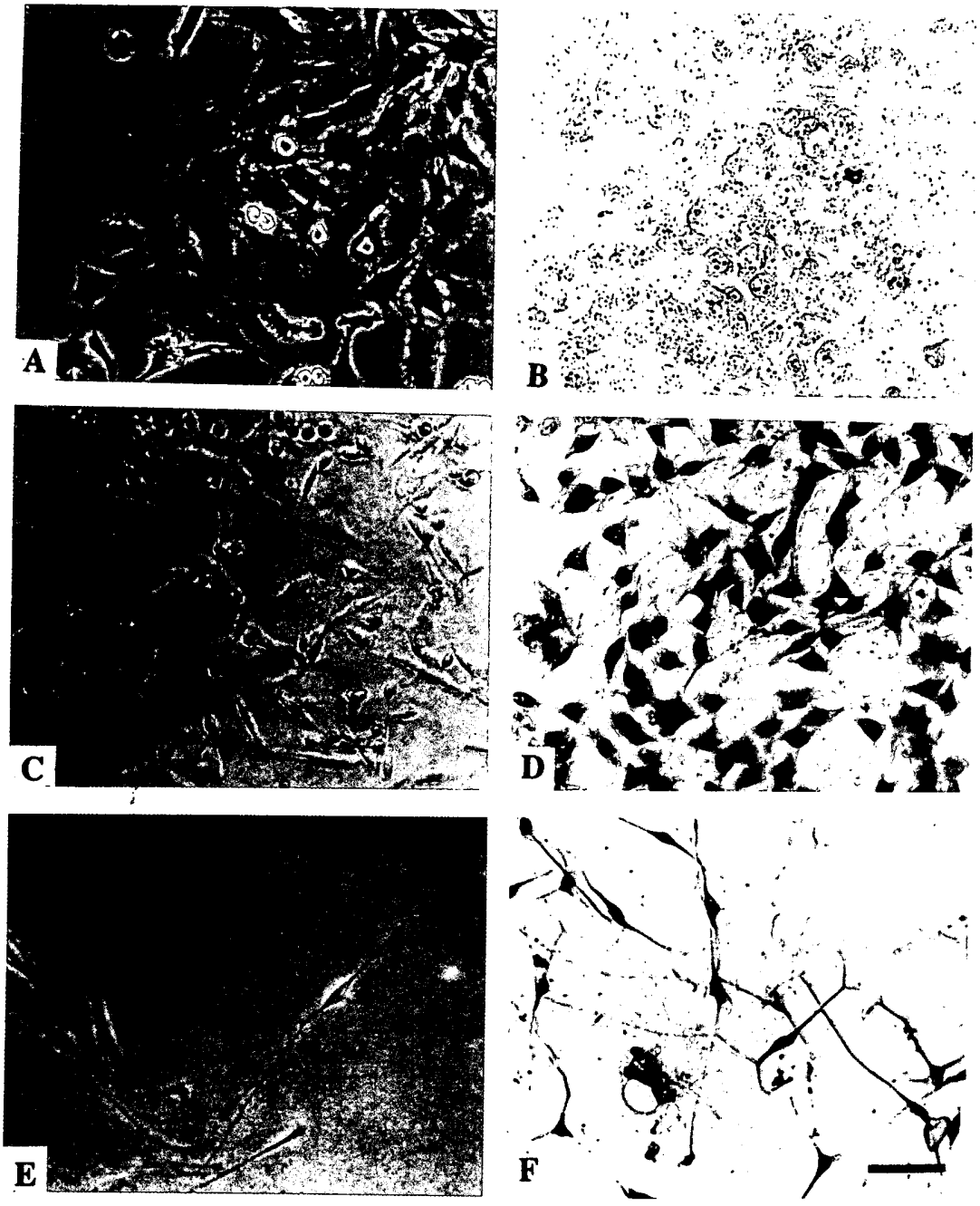

Fig. 2. Morphology of immortalized olfactory cell lines. Phase-contrast micrographs of unstained (A,C,E) or Nissl stained (B,D,F) cultures. **A,B:** OLF4.7.8, a Class I cell line. **C,D:** OLF4.4.1, a Class II cell line. **E,F:** OLF 4.3, a Class III cell line. Scale bar = 50 μm.

the cell lines the OMP probe hybridised with three mRNA transcripts but in the olfactory epithelium and bulb only a single transcript was observed.

No data were available on the sequence of the mouse G_{OLF} gene, consequently Northern analysis was performed using a full length, rat G_{OLF} cDNA probe. As controls, total mRNA was extracted from adult rat olfactory epithelium and the following tissues from adult mouse: olfactory epithelium, olfactory bulb, and kidney. As expected, rat olfactory epithelium expressed two mRNA transcripts, 2.7 and 3.5 kb (Jones and Reed, 1989), confirming the efficacy of the cDNA probe (Fig. 6B). In contrast, mouse olfactory epithelium expressed two mRNA transcripts of 2.7 and 6 kb. Neither mouse

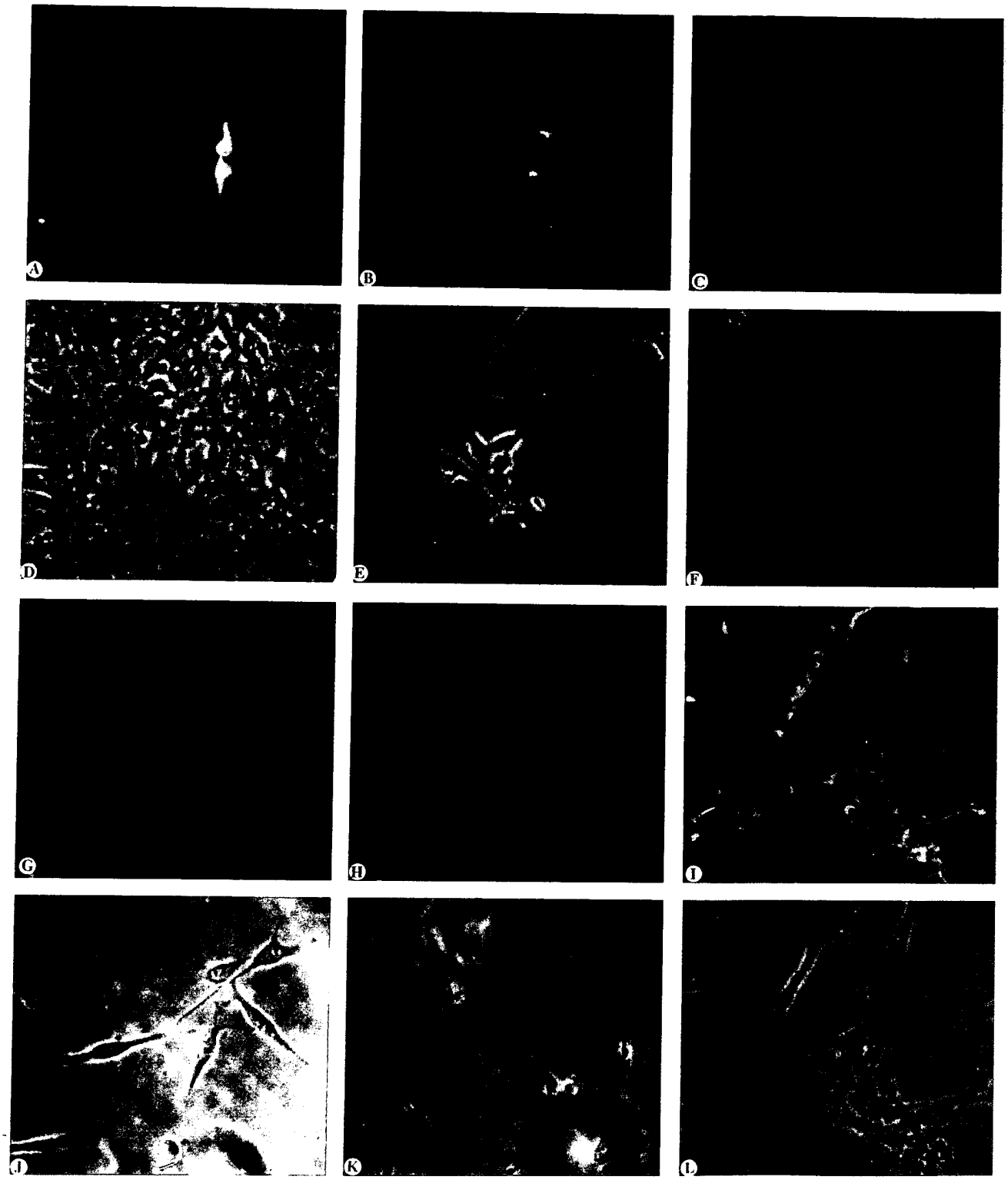

Fig. 3. Intermediate filament expression. Neurofilament staining in (**A,D**) OLF4.7.8 a Class I cell line, (**B,E**) OLF10.1.8 a Class II cell line and (**C,F**) OLF4.3 a Class III cell line. GFAP staining in (**G,J**) OLF4.4.2 a Class II cell line and (**H,K**) OLF4.1 a Class III cell line. **I,L:** Vimentin staining in OLF4.5.1 a Class I cell line. A, B, C, G, H, I, fluorescent micrographs (200×). D, E, F, J, K, L, phase-contrast images of the same fields.

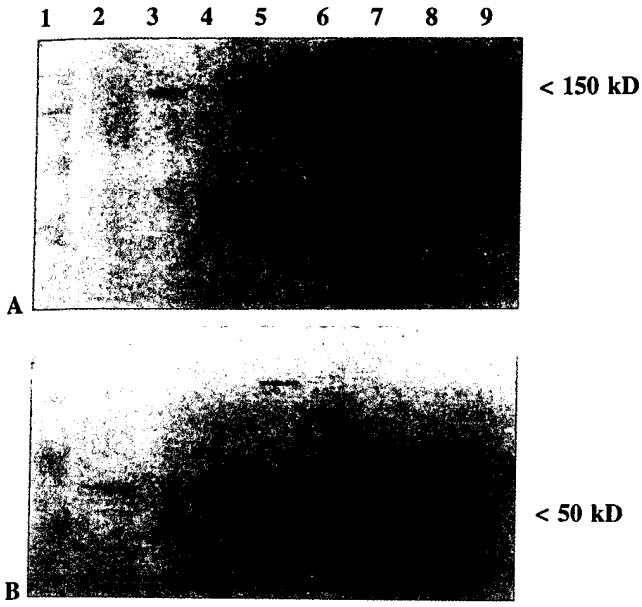

Fig. 4. Immunoblot analysis of neurofilament and GFAP immunoreactivity. Immunoblots probed with (**A**) neurofilament antiserum (1:400) or (**B**) antiserum to GFAP (1:100). Lane markings are: 1, molecular weight markers (top to bottom; 205 kD, 116.5 kD, 80 kD and 49.5 kD); 2, olfactory epithelium; 3, OLF3.6 (Class III); 4, OLF4.1 (Class III); 5, OLF4.3 (Class III); 6, OLF4.4.1 (Class II); 7, OLF4.4.2 (Class II); 8, OLF4.5.1 (Class I); 9, OLF4.7.8 (Class I). Indicated beside each photograph is the molecular weight of the antigens.

kidney nor olfactory bulb expressed either transcript (Fig. 6B). Northern analysis of purified mRNA revealed that in each of the five cell lines screened, the 2.7 kb G_{OLF} transcript, but not the 6 kb transcript was expressed. Due to the abundance of G_{OLF} mRNA in the olfactory epithelium, distinct bands could not be discerned in the lane corresponding to this tissue (Fig. 6C). The absence of a band corresponding to 2.7 kb in the lane corresponding to the olfactory epithelium is a result of a reversal of the image due to extreme overexposure.

DISCUSSION

Twenty-one cell lines were generated by immortalisation of neuronal precursors from the olfactory epithelium of the adult mouse. These cell lines fell into three broad classes, based on their morphology, and intermediate filament protein expression and cell lines in each class were of similar phenotype. Some of the cell lines expressed mRNA transcripts for OMP, a protein expressed mainly by mature olfactory sensory neurons, and G_{OLF}, a G-protein involved in olfactory sensory transduction. The phenotypes of these different classes of cell lines were very stable and the same mixture of cell types within each cell line is present after several years of passage. The morphologies within each cell line apparently represent different stages of differentiation. For example all cells in Class II cell lines can be induced into bipolar neuronal morphologies by serum deprivation allowing the identification of known and novel genes involved in differentiation (Zehntner et al., 1996). Some of the cell lines reported here showed similar morphologies to cell lines generated from the olfactory epithelium by a different technique (Largent et al., 1993). In that case the cell lines were isolated from an olfactory neuroepithelial tumour which was produced in transgenic animals by placing the expression of an oncogene (the simian virus 40 T-antigen) under the regulatory elements of OMP (Largent et al., 1993). It is interesting to note that both approaches led to cell lines representing neuronal precursors rather than mature neurons. In the present study this was expected because retroviral insertion of the oncogene can take place only in dividing cells whereas it might be expected that genes under OMP-regulatory elements would be expressed by cells with the mature phenotype.

Origin of Immortalized Cell Lines

Using the techniques described here, *n-myc* is incorporated only into the cells which were undergoing division during the period of transfection. Within the olfactory epithelium there are two different cell populations undergoing division: the supporting cells and the basal cells. Although these two populations are of similar density within the olfactory epithelium (2×10^4 per mm^2; Mackay-Sim et al., 1988), the rate of cell division within the basal cells is normally about 16 times the rate of supporting cell division, as judged by the numbers of cells labelled after a single injection of ^{3}H-thymidine (Mackay-Sim and Beard, 1987). This ratio was increased even further by removing the olfactory bulbs of the donor animals 4 days prior to removal of the olfactory epithelium, a period during which basal cell division is increased 12-fold over control. It is therefore estimated that the rate of cell division in the basal cells was close to 200 times greater than that of the supporting cells at the time of immortalisation. By force of probability we conclude that it was the basal cells which were immortalized by the *n-myc* transfection technique used here.

The basal cells of the olfactory epithelium are a heterogeneous population of dark and globose cells both of which undergo division (Andres, 1966; Calof and Chikaraishi, 1989; Caggiano et al., 1994; Satoh and Takeuchi, 1995; Graziadei and Monti Graziadei, 1979). The globose basal cells are themselves heterogeneous undergoing several divisions before differentiating into sensory neurons (Mackay-Sim and Kittel, 1991; Caggiano et al., 1994). Given this heterogeneity among the

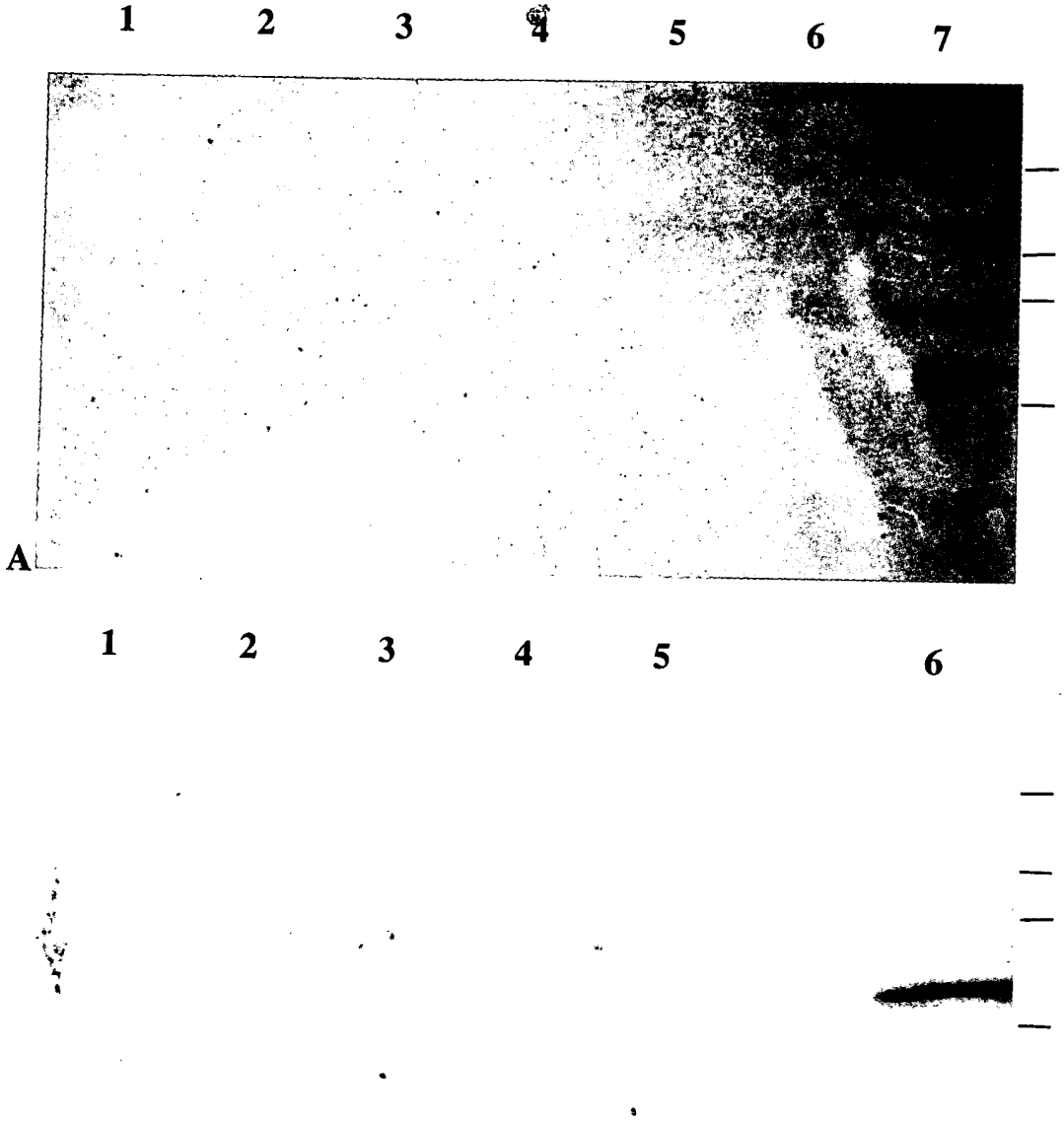

Fig. 5. Immunoblot analysis of OMP expression. Immunoblot blots probed with an OMP antiserum (1:500). **A:** Lane markings are: 1, OLF4.4.2 (Class II); 2, OLF4.4.1 (Class II); 3, OLF4.3 (Class III); 4, OLF4.1 (Class III); 5, OLF3.6 (Class III); 6, OLF3.3 (Class III); 7, olfactory epithelium. **B:** Lane markings are: 1, OLF4.7.8 (Class I); 2, OLF4.6.3 (Class II); 3, OLF4.6.2 (Class II); 4, OLF4.6.1 (Class II); 5, OLF4.5.1 (Class I); 6, olfactory epithelium. For both blots, markings on the side of the blots indicate molecular weights of 49.5 kD, 32.5 kD, 27.5 kD and 18.5 kD (top to bottom).

basal cells, any of which might have been immortalized, it was predicted that the transfection procedure might produce a heterogeneous population of immortalized cell lines. This turned out to be the case, with three main morphological classes of cell lines whose intermediate filament expression was similar within each class but different between classes.

Vimentin is an intermediate filament protein which is expressed during development in proliferating neuroepithelium and neural crest (Schnitzer et al., 1981; Ziller et al., 1983). Normally postmitotic neurons cease expressing vimentin and begin to produce neurofilament proteins although there is a brief period during which both vimentin and neurofilaments coexist in immature neurons or their precursors (Cochard and Paulin, 1984). Vimentin is expressed by olfactory sensory neurons and their precursors (Schwob et al., 1986; Gorham et al., 1991). The expression of neurofilament proteins in the

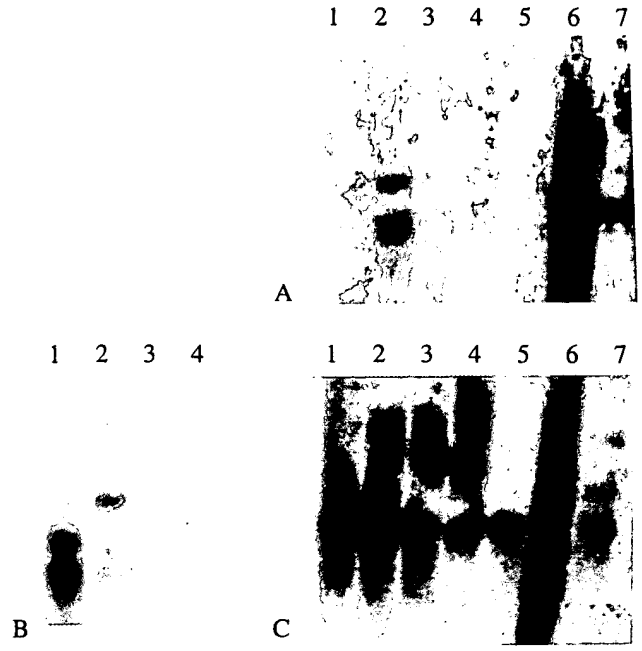

Fig. 6. Expression of OMP and Golf mRNA in olfactory tissue and the immortalized cell lines. **A:** Random hexamer-primed OMP cDNA fragment, labelled with ^{32}P, was used to probe a Northern blot of poly(A^+) RNA (5 μg/lane) from mouse olfactory epithelium and bulb, and five immortalized cell lines. Lane markings are: 1, 4.4.1 (Class II); 2, 4.4.2 (Class II); 3, 4.6.1 (Class II); 4, 4.6.3 (Class II); 5, 4.5.1 (Class I); 6, olfactory epithelium; 7, olfactory bulb. **B:** Northern blot of total RNA (20 μg/lane) from rat olfactory epithelium (Lane 1), mouse olfactory epithelium (Lane 2), mouse olfactory bulb (Lane 3) and mouse kidney (Lane 4) probed with a ^{32}P-labelled Golf cDNA fragment. **C:** Northern blot of poly(A^+) RNA (5 μg/lane) from mouse olfactory epithelium and bulb, and five immortalized cell lines probed with a ^{32}P-labelled Golf cDNA fragment. Lane markings are as in A.

olfactory epithelium is moot, with some authors observing expression and others not (Vollrath et al., 1985; Trojanowski et al., 1991; Schwob et al., 1986; Talamo et al., 1989) but these differences may depend upon the phosphorylation state of the neurofilament protein and the antibody used (Talamo et al., 1989) as well as fixation, species or age differences. Neurofilament expression is readily observed in primary cultures of olfactory epithelium (Chuah et al., 1991; MacDonald et al., 1996). All the cell lines expressed vimentin, as would be expected of cells derived from the neuronal precursors of the olfactory epithelium. Classes II and III also expressed neurofilament. Additionally Class III cell lines also expressed GFAP, confirmed by immunoblot analysis. This would not normally be expected of neuronal precursors in the olfactory epithelium.

The flat morphology and lack of neurofilament expression in the Class I cell lines suggest that they may be derived from the keratin-positive basal cells in the olfactory epithelium. Their morphology is similar to keratin-positive cells in primary culture (MacDonald et al., 1996; Pixley, 1992b; Satoh and Takeuchi, 1995) and Class I cell lines do express keratin (MacDonald, unpublished observations). The mixed morphology of the Class II cell lines and their expression of neurofilament protein suggest that they may be derived from globose basal cells which are a heterogeneous population containing dividing cells at different stages of the lineage pathway (Mackay-Sim and Kittel, 1991; Caggiano et al., 1994). Although the globose basal cells have not been shown to express neurofilament in vivo, this protein is expressed by a dividing population of olfactory epithelial cells in vitro (MacDonald et al., 1996). The origin of Class III cell lines is more difficult to surmise since they express both neurofilament and GFAP. GFAP is expressed by the olfactory nerve ensheathing cells (Barber and Lindsay, 1982; Pixley, 1992a; Ramón-Cueto and Nieto-Sampedro, 1992) and there is some evidence that they derive from the olfactory epithelium (Chuah and Au, 1991). Furthermore, Akeson and Haines (1989) found GFAP-positive cells in cultures of dissociated neonatal rat olfactory epithelium. This raises the possibility that Class III cell lines may derive from glial precursors in the olfactory epithelium. Similarly, both GFAP and neurofilament protein are expressed in human glioma cell lines (Tohyama et al., 1993). Alternatively, it is possible that *n-myc* transfection has revealed a multipotent precursor cell resident in the olfactory epithelium which can give rise to both neurons and glia. Immortalized cell lines derived from embryonic mouse neuroepithelium (Bartlett et al., 1988) and embryonic rat cerebellum (Frederiksen et al., 1988) are multipotent and can differentiate into cells with either neuronal or glial properties.

Some of the Cell Lines Partially Differentiate

The immortalized cell lines of Class II seem to represent globose basal cells, the population of neural precursor/progenitor cells. As indicated this population, in vivo, is expected to be heterogeneous, at different stages along the lineage pathway. In accordance with this hypothesis, some of these cell lines express markers of mature sensory neurons, while others do not. The markers in question are OMP and G_{OLF}.

OMP immunoreactivity is observed in the cell bodies and dendrites of olfactory sensory neurons in the olfactory epithelium and in their axons in the olfactory bulb (Baker et al., 1989). OMP mRNA is also observed the cell bodies of olfactory sensory neurons and their axons in the olfactory bulb (Ressler et al., 1994). OMP expression is generally associated with mature sensory neurons (Baker et al., 1989) although it has been shown

in immature olfactory neurons (Rogers et al., 1987). It is also present in olfactory organ cultures in which full sensory neuron maturity cannot occur, that is, in the absence of the olfactory bulb (Chuah and Farbman, 1983). In the present study OMP mRNA transcripts were expressed in the four Class II cell lines which were examined but not in the Class I cell line (Fig. 5).

G_{OLF}, an olfactory-specific G-protein, is one of two stimulatory G-proteins expressed in the olfactory epithelium. The G_{OLF} protein is abundant in the olfactory epithelium, and is the only stimulatory G-protein present in the cilia of the olfactory neurons (Jones, 1990). Low level expression of G_{OLF} mRNA was detected by Northern analysis of purified mRNA in five cell lines. Of these, four were from Class II and one was from Class I (Fig. 6). In the cell lines and the mouse olfactory epithelium, a single 2.7 kb mRNA transcript hybridised with the probe, which was based on the sequence for the rat G_{OLF} mRNA. The same probe hybridised with an additional 3.5 kb mRNA transcript in RNA purified from rat olfactory epithelium (Fig. 6). These latter results confirm the observations of two transcripts from rat olfactory epithelium (Jones and Reed, 1989). There are no published reports on mouse G_{OLF} but our results suggest that its sequence is not identical to the rat.

The discovery of OMP and G_{OLF} expression in the Class II cell lines provides further evidence of their olfactory origin and suggests that some of the cells are partially differentiated. Since these are dividing cell populations of cells it is to be expected that the cells will distributed around the cell cycle with only a proportion of them partially differentiated. This could explain the low levels of expression of OMP and G_{OLF} in these populations. The expression of these sensory neuron markers suggests that these cell lines may have been immortalized from sensory neuron precursors undergoing a final mitosis before terminal differentiation. This makes these cell lines important targets for further research. They provide an unlimited supply of genetically homogeneous cells for genetic analyses of neuronal differentiation (Zehntner et al., 1996). Of great interest are the observation that differentiation induces changes in the expression of membrane ion currents (Cuffel et al., 1994) and expression of olfactory receptor genes (Dowsing et al., 1995).

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